Supplement to

Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning

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SUPPLEMENTAL TABLES

Citation numbers in Tables S1-S6 refer to the bibliography at the end of this supplement.

Table S1. Pro-angiogenic and anti-angiogenic effects of proteases

Experimental studies reporting positive and negative effects of proteases on functional vessel growth. EC: endothelial cell; BAEC: bovine adrenal-cortex derived EC; HUVEC: human umbilical vein EC; OIR: oxygen-induced retinopathy.

Ref	System	Mode of observation	Observations
Proteases hindering functional vessel growth			
(1)	Bovine adrenal cortical ECs	EC proliferation	Plasmin cleaved $VEGF_{165}$ reduces cell proliferation (EC50) by ~100-fold.
(2)	Tumor xenograft	Tumor volume	Cleaved VEGF results in weak tumor growth, compared to $VEGF_{164}$.
(3)	Mouse retina oxygen-induced retinopathy	Vascular morphology	VEGF cleavage induces vascular malformations, compared to absence of cleavage.
(4) (5) (6)	Chronic venous leg ulcer Diabetic mouse wound Tumor xenograft	SDS-PAGE Wound closure kinetics Vessel growth	VEGF degradation by proteases results in decreased levels of active VEGF and decreased angiogenesis response. Degradation by plasmin leads to decreased angiogenesis and wound closure rates.
Proteases inducing functional vessel growth			
(7) (8)	HUVEC culture Tumor xenograft	Vessel morphology Tumor volume	Proteases degrade VEGF inhibitors (e.g. CTGF, sVEGFR1) to induce angiogenesis
(9) (10)	BAEC culture HUVEC	EC proliferation EC proliferation	Cleavage of $VEGF_{183}$/$VEGF_{189}$ or heparin co-presentation activates mitogenic behavior of these isoforms.
(11)	Matrix-tethered VEGF + flow	Capillary formation	Proteases induce VEGF gradients and capillary morphogenesis
(12)	Colon tumor explants	Vessel growth	Proteases cleave HSPG core protein to liberate VEGF, induce angiogenesis
(13) (14) (15) (16) (17)	Pancreatic islet tumor explants Breast tumor mouse Breast tumor mouse Glioblastoma mouse Cervical cancer mouse	Tumor volume Tumor volume Tumor volume Vessel morphology Tumor volume	In most systems, MMP9 liberates VEGF and induces angiogenesis and/or tumor growth. In glioblastoma, MMP9 release of VEGF increases pathological angiogenesis but this impedes perivascular tumor invasion.

Table S2. Experimental observations of VEGF gradients

Experimental studies visualizing VEGF gradients and the spatial range of VEGF isoforms.

Ref	System	Mode of observation	Observations of studies
Visualization of VEGF gradients through immunostaining			
(18) (19) (20)	Mouse hindbrain Mouse retina Mouse cerebellum	Immunostaining Immunostaining Immunostaining	Heparin binding isoforms distribute near origin of secretion and show localization to cell surfaces with little in interstitium. $VEGF_{120}$ is noted to have higher concentrations at a distance from source and shows intense staining in the interstitium.
(21)	Zebrafish	Immunostaining	Loss of perlecan results in more widespread VEGF distribution and increased VEGF concentrations.
(22) (23) (24)	Tumor cell culture Tumor xenograft Bruch's Limiting Membrane *ex vivo*	Immunostaining Immunostaining Immunostaining	In xenograft, different VEGF isoforms show similar binding to endothelial cells and heparin-affinity dependent binding to tumor cells. $VEGF_{120}$ staining is nearly absent from ECM or tumor cells in all three systems.
Spatial range of vascular effects of VEGF isoforms and released forms			
(23) (2) (25) (26) (27)	Tumor xenograft Tumor xenograft Rabbit cornea Rabbit cornea CAM Assay	Vessel morphology Vessel morphology Angiogenesis Angiogenesis Cell Proliferation	$VEGF_{120}$ is more effective in activating peritumoral vasculature than $VEGF_{188}$. $VEGF_{188}$ and protease-resistant VEGF have weaker peritumoral effects. $VEGF_{121}$ shows earlier onset and/or greater angiogenesis in rabbit corneal assay. $VEGF_{121}$ had greater range in CAM assay. Vascular effects in CAM were mediated by intussusceptive angiogenesis.
(6)	Tumor xenograft	Vessel morphology	Nondegradable VEGF has greatest spatial range ($VEGF_{121} < VEGF_{165} < VEGF_{111}$).
(13) (3)	Pancreatic islet explant Mouse OIR	Vessel activation Vessel morphology	MMP9-released VEGF induces new vessel sprouts. VEGF cleavage by MMP12 induces vascular malformations in nascent vessels.
(28)	Mouse epithelial bud	Bud morphology	FGF10 (matrix binding form) results in a localized proliferation compared to FGF7 (diffusible form).

Table S3. Experimental observations of soluble and matrix-/receptor-bound VEGF levels

Ref	System	Mode of observation	Observations of studies
Levels of soluble VEGF			
(29)	Eye	ELISA	Elevated levels of intraocular and serum VEGF in systems secreting non-heparin-binding ($VEGF_{120}$ and $VEGF_{113}$) isoforms relative to $VEGF_{164}$, $VEGF_{188}$ systems.
(2)	Tumor xenografts, serum	ELISA	
(6)	Tumor xenografts, serum	ELISA	No difference in serum concentration between tumors secreting different isoforms
(30)	Tumor cell culture	Conditioned medium (CM) + Heparin, ELISA	Soluble VEGF in $VEGF_{120}$ > $VEGF_{165}$ > $VEGF_{189}$ secreting cells. Bound fraction increases with isoform.
(26)	Tumor cell culture		
Levels of immunohistochemically or immunofluorescently stained VEGF			
(24)	Bruch's Limiting Membrane	Immunostaining	Peritumoral and ECM staining of VEGF directly correlated with VEGF isoform's heparin binding affinity. $VEGF_{120}$-secreting systems show no parenchymal or ECM staining (only vascular staining) while $VEGF_{188}$-secreting systems show strong interstitial staining. VEGF has strong localization when MMP9 is weakly active and disperse staining when MMP9 is highly active.
(22)	Tumor Cell culture	Immunostaining	
(23)	Tumor xenografts	Immunostaining	
(14)	Tumor xenografts	Immunostaining	
(18)	Mouse hindbrain	Immunostaining	In contrast to above studies, $VEGF_{120}$-secreting systems show strong VEGF staining, similar in magnitude to wildtype systems.
(19)	Mouse retina	Immunostaining	
(20)	Mouse cerebellum	Immunostaining	
Levels of vascular receptor-bound VEGF			
(23)	Tumor xenograft	Immunostaining	All isoforms yield strong VEGF binding to vessels. Autocrine VEGF secretion by vessels may contribute.
(13)	Pancreatic islet tumor	Immunostaining	Increased intensity of VEGF-VEGFR2 staining in systems that contain high levels of active protease.
(14)	Breast cancer	Immunostaining	
(15)	Breast cancer	Immunostaining	
(17)	Cervical cancer	Immunostaining	
(16)	Glioblastoma	Immunostaining	
(3)	Oxygen-induced retinopathy	Immunostaining	

Table S4. Experimental observations of total VEGF levels

Ref	System	Mode of observation	Observations of studies
Levels of total VEGF			
(30) (31) (26)	Tumor cell culture Tumor cell culture Tumor cell culture	CM+heparin, ELISA CM+suramin, ELISA CM+suramin, ELISA	In tumor cell cultures, total level of VEGF, after liberation of bound VEGF by heparin or suramin, is similar between cells transfected with different isoforms.
(31) (26)	Tumor xenograft Tumor xenograft	Tissue lysate ELISA Tissue lysate ELISA	In contrast to *in vitro* culture, total VEGF levels in tumors are significantly higher in $VEGF_{164}$-secreting tumors than in $VEGF_{120}$-secreting tumors.
(21)	Zebrafish	Immunoblotting	Total VEGF is higher in zebrafish embryos with perlecan knockdown
(14) (15) (16, 32)	Breast cancer Breast tumor xenograft Glioblastoma (WT vs. MMP9-KO)	Tissue ELISA/enzyme immunoassay	Total VEGF levels are not affected by proteolytic release.
(33) (32)	Meta-analysis Whole mouse study	Modeling Real time RT-PCR /serum ELISA	Analysis suggests most tissue VEGF is located intracellularly. Endothelial intracellular VEGF is a negligible fraction of this total VEGF.

Table S5. Relative isoform expression in various organs and tumors.
The data in this table was used to generate Figure 2 in this manuscript. 121: $VEGF_{121}$; 165: $VEGF_{165}$; Ex6: Exon 6-containing isoforms. WB: Western blot. *: RT-PCR bands analyzed using ImageJ (NIH). When ranges were provided, the midpoint was used for Figure 2.

Ref	% 121	% 165	% Ex6	System	Tissue	Method
(34)	37	23	40	Tumor, human	Bladder cancer, <=pT1	RT-PCR
(34)	41	23	36	Tumor, human	Bladder cancer, >pT1	RT-PCR
(35)	~40	~60	~0	Embryonic mouse	Bone	qRT-PCR
(36)	57	31	11	Embryonic mouse	Bone	qRT-PCR
(37)	~33	~37	~30	Tumor culture	U87MG cell line (Brain)	WB, RT-PCR*
(38)	18	70	12	Adult mouse	Brain	RT-PCR
(39)	14	80	6	Adult mouse	Brain	RT-PCR
(20)	16	81	3	Embryonic mouse	Cerebellum	RT-PCR
(40)	48	45	7	Tumor culture	BT20 cell line (Breast)	RT-PCR
(40)	49	41	10	Tumor culture	MCF-7 cell line (Breast)	RT-PCR
(40)	46	45	9	Tumor culture	MDA-MB-231 cell line (Breast)	RT-PCR
(40)	64	35	1	Tumor culture	MDA-MB-453 cell line (Breast)	RT-PCR
(40)	55	40	5	Tumor culture	T-47D cell line (Breast)	RT-PCR
(40)	72	28	0	Tumor culture	MCF-12A cell line (Breast)	RT-PCR
(41)	~56	~43	~1	Tumor, human	Colon	RT-PCR*
(41)	~78	~22	~0	Normal human	Colon, non-tumor tissue	RT-PCR*
(42)	4	95	1	Embryonic, mouse	Eye (lens)	qPCR
(39)	26	69	5	Adult mouse	Eye	RT-PCR
(38)	7	91	2	Adult mouse	Retina	RT-PCR
(38)	25	75	0	Adult mouse	Choroid/Retinal pigment epithelium	RT-PCR
(39)	11	53	36	Adult mouse	Heart	RT-PCR
(43)	3	29	68	Normal rat	Heart	RT-PCR
(44)	5	59	35	Normal mouse	Heart	qRT-PCR
(45)	19	59	22	Normal human	Right atrium	qRT-PCR
(46)	70	30	0	Tumor, human	Renal cell cancer	Competitive RT-PCR
(39)	20	63	17	Adult mouse	Kidney	RT-PCR
(43)	18	51	31	Normal rat	Kidney	RT-PCR
(44)	37	48	15	Normal mouse	Kidney	qRT-PCR
(39)	9	55	36	Adult mouse	Liver	RT-PCR
(44)	30	47	23	Normal mouse	Liver	qRT-PCR
(47)	68	24	8	Tumor, human	NSCLC (median values)	RT-PCR
(48)	73	27	0	Tumor, human	NSCLC	RT-PCR
(48)	71	28	1	Normal human	NSCLC adjacent tissue	RT-PCR
(41)	~69	~28	~3	Tumor, human	Lung	RT-PCR*
(41)	~43	~8	~49	Normal human	Lung, non-tumor tissue	RT-PCR*
(49)	50	40	10	Embryonic rat	Lung	RT-PCR
(49)	25	25	50	Adult, rat	Lung	RT-PCR
(39)	18	30	52	Embryonic mouse	Lung	RT-PCR
(44)	29	24	47	Normal, mouse	Lung	qRT-PCR
(38)	9	22	69	Adult, mouse	Lung	RT-PCR
(39)	8	77	15	Adult mouse	Muscle	RT-PCR
(43)	6	51	43	Normal, rat	Muscle	RT-PCR
(50)	19	46	35	Normal, rat	Muscle	RT_PCR
(40)	47	42	11	Tumor culture	OVCAR-3 cell line (Ovary)	RT-PCR
(40)	70	25	5	Tumor culture	SK-OV-3 cell line (Ovary)	RT-PCR
(39)	42	53	5	Adult mouse	Ovary	RT-PCR
(51)	23	63	15	Normal, human	Prostate, normal	qRT-PCR
(51)	45	46	8	Tumor, human	Prostate, cancer	qRT-PCR
(39)	45	48	7	Adult mouse	Skin	RT-PCR
(52)	52-70	26-42	4-8	Tumor, human	Melanoma	RT-PCR
(53)	56	33	11	Tumor, human	HNSCC + node metastasis	RT-PCR
(53)	61	28	11	Tumor, human	HNSCC, no node metastasis	RT-PCR
(53)	53	33	14	Normal, human	Normal tonsil mucosa	RT-PCR

Table S6. Experimental studies illustrating atypical phenotypes of VEGF isoforms

Experimental studies that report observations at odds with the typical monotonic ordering of vascular phenotypes vs. isoform size and matrix affinity. Most comparisons are made between transgenic systems constructed to express only one isoform, at a constant secretion rate. Comparisons are not made between the levels of the different VEGF isoforms in a single system expressing multiple isoforms simultaneously, e.g. wildtype hindbrain.

Ref	System	Mode of observation	Observations of studies
VEGF121 is more angiogenic and tumorigenic than VEGF165			
(25)	Rabbit cornea	Vessel imaging	$VEGF_{121}$ implants induced greater angiogenesis than $VEGF_{165}$ implants
(26)	Tumor xenograft	Tumor growth	$VEGF_{121}$ induced faster angiogenesis and tumor growth than $VEGF_{165}$ or $VEGF_{189}$
VEGF188 vs. VEGF164Δ108-118 vs. VEGF111			
(23)	Tumor xenograft	Tumor growth kinetics	$VEGF_{188}$: thin, hypervascular angiogenesis and no potentiation of tumor growth
(2)	Tumor xenograft	Tumor growth kinetics	$VEGF_{164\Delta 108\text{-}118}$: thin, hypervascular angiogenesis and very strong potentiation of tumor growth
(6)	Tumor xenograft	Vascular phenotype	$VEGF_{111}$ (resistant to proteolysis): thin, hypervascular angiogenesis periturmorally, decreased vascularization intratumorally. Protease resistance rather than matrix binding may be cause for hypervascular networks.
VEGF isoforms showing similar vascular patterning			
(54) (19)	Mouse skeletal muscle Mouse retina	Vessel morphology Vessel morphology	Under conditions of lens- and myoblast-overexpression, $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ induce the same malformations to existing vasculature.
(18)	HUVEC culture	Cell proliferation	$VEGF_{120}$ and $VEGF_{164}$ induce same proliferatory response.

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